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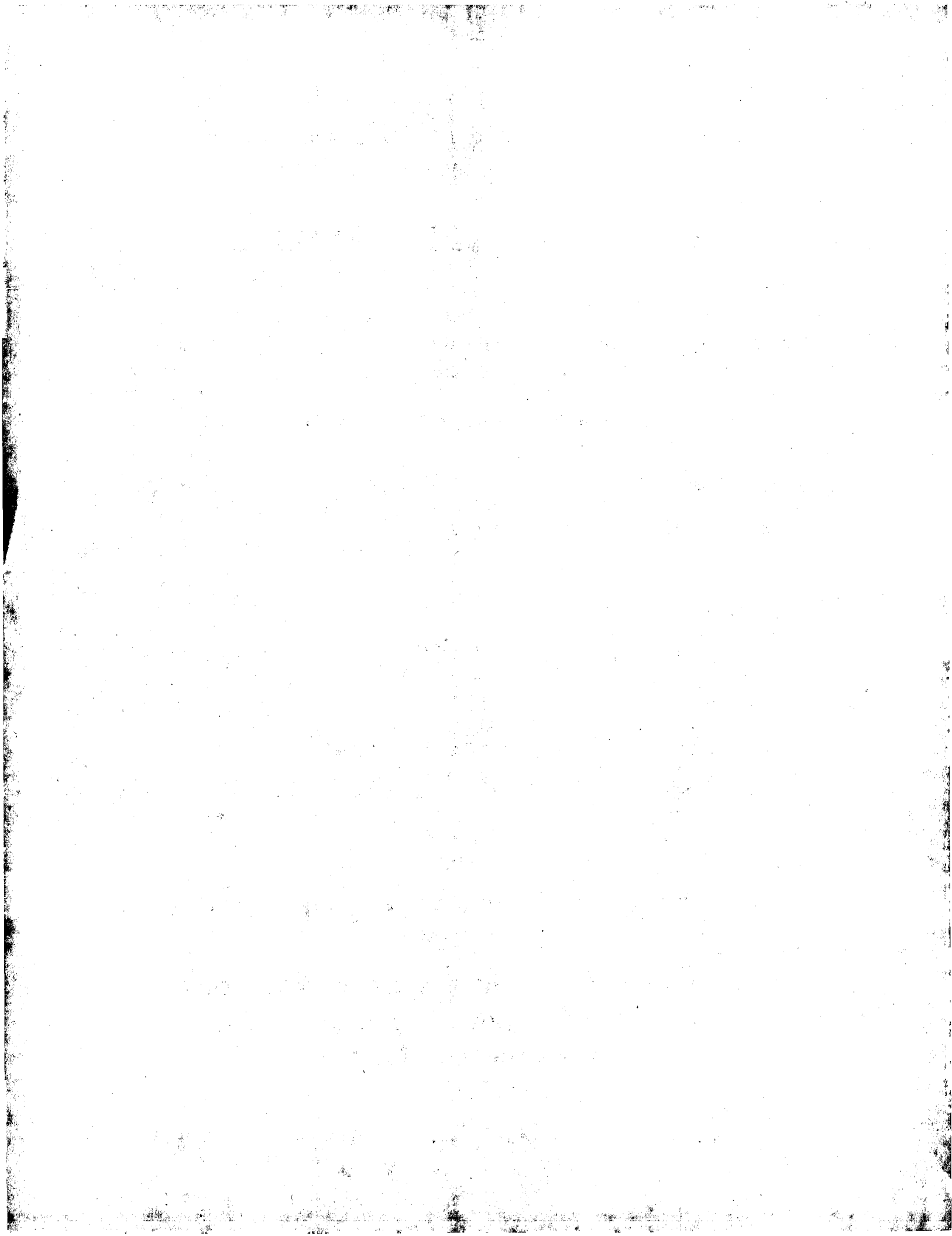
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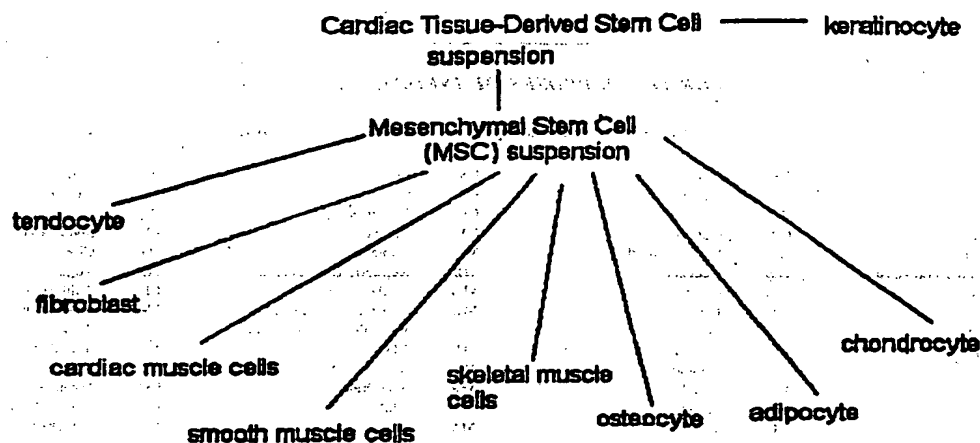
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(54) Title: CARDIAC-DERIVED STEM CELLS



(57) Abstract

The invention provides cardiac-derived pluripotent stem cells, which on proliferation and differentiation can produce a variety of cell types including cardiocytes, fibroblasts, smooth muscle cells, skeletal muscle cells, keratinocytes, osteoblasts and chondrocytes. The cells can be used in methods of treating patients suffering from necrotic heart tissue. The stem cells proliferate and differentiate to produce cardiocytes replacing the necrotic tissue. The cells can also be used to screen compounds for activity in promoting proliferation and/or differentiation of cardiac-derived stem cells.

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CARDIAC-DERIVED STEM CELLS**CROSS-REFERENCES TO RELATED APPLICATIONS**

- 5 This application derives priority from USSN 60/079,123, filed on March 23, 1998, which is incorporated by reference in its entirety for all purposes.

TECHNICAL FIELD

- The invention resides in the technical fields of cell biology, drug discovery
10 and medicine.

BACKGROUND OF THE INVENTION

- In several tissues, the terminal state of cell differentiation is incompatible with cell division. For example, in outerlayer skin cells, the cell nucleus is disintegrated,
15 in red blood cells, there is no nucleus, and in muscle cells, myofibrils obstruct mitosis and cytokinesis. The ability of such cells and tissues to develop, regenerate and repair is dependent on the existence of stem cells that on division form additional differentiated progeny cells.

- Stem cells are not terminally differentiated, can divide without limit, and
20 give rise to progeny, which can continue to divide or can differentiate. Stem cells can be totipotent, pluripotent or unipotent. Totipotent stem cells (e.g., embryonic stem cells) can give rise to every cell type in an adult organism. Pluripotent stem cells can give rise to more than one differentiated cell type. A unipotent stem cell can give rise to a single differentiated cell type. Stem cells are generally characterized by small size, low
25 granularity, low cytoplasmic to nuclear ratio and no expression of osteopontin, collagens and alkaline phosphatase. The existence of stem cells is well-documented for epidermis, intestinal epithelial and hematopoietic systems. Hemopoietic stem cells are present in circulation as well as bone marrow. Circulating hemopoietic stem cells can colonize organs such as spleen. Stem cells for cells of bone, cartilage, fat and three types of
30 muscle (smooth, skeletal and cardiocyte) are thought to be a common mesenchymal stem cell precursor, but neither mesenchymal stem cells or the more committed tissue specific progenitors have been characterized (Owen et al., *Ciba Fdn. Symp.* 136, 42-46, 1988); Owen et al., *J. Cell Sci.* 87, 731-738 (1987)).

- Zohar, *Blood* 90, 3471-3481 (1997) reports attempts to identify progenitors of osteogenic cells by isolating cells from fetal rat periosteum and screening for differential expression of markers, protein content and cell cycle position. Huss et al., *PNAS* 92, 748-752 (1995) report the identification of a stromal cell precursor from canine bone marrow culture. The precursor was reported to differentiate in the presence of growth factor and display mature differentiation markers. Robbins et al., *Trends Cardiovasc. Med.* 2, 44-50 (1992) report that mouse embryonic stem cells can differentiate into embryoid bodies that simulate some aspects of cardiogenesis.

10 DEFINITIONS

- An isolated cell is a cell that has been at least partially purified from other cell types with which it is naturally associated. Often an isolated cell exists in a population of cells at least 25, 50, 75, 90, 95 or 99% of which are the isolated cell type. Sometimes an isolated cell gives rise to a cell line in which all cells are essentially identical except for spontaneous mutations that may arise in propagation of the cell line. Sometimes an isolated cell undergoes spontaneous differentiation to generate a mixed population of mature cell types.

- A set of differentiation markers means one or more phenotypic properties that can be identified and are specific to a particular cell type. Differentiation markers are transiently exhibited at various stages of cell lineage. Pluripotent stem cells that can regenerate without commitment to a lineage express a set of differentiation markers that may be lost when commitment to a cell lineage is made. Precursor cells express a set of differentiation markers that may or may not continue to be expressed as the cells progress down the cell lineage pathway toward maturation. Differentiation markers that are expressed exclusively by mature cells are usually functional properties such as cell products, enzymes to produce cell products and receptors.

- Adherence capacity of cells in culture is one marker of progression from an undifferentiated to differentiated state. Adherent cells form a monolayer on plastic substrate.

- 30 A cell lineage refers to a differentiated cell type and the ancestor cell types from which the differentiated cell type was derived. For example, cardiocytes and myoblasts are two cell types in the same lineage.

SUMMARY OF THE INVENTION

In one aspect, the invention provide isolated nonadherent pluripotent cardiac-derived human stem cells. On proliferation and differentiation such cells produce progeny cells comprising a cell type selected from the group consisting of an adherent cardiac-derived stem cell, a fibroblast, a smooth muscle cell, a skeletal muscle cell, a cardiocyte, a keratinocyte, an osteoblast and a chondrocyte. Some such stem cells generate all of the cell types selected from the group. Nonadherent cardiac-derived stem cells of the invention are producible by propagating a population of heart tissue-derived cells in a liquid medium on a substrate; and discarding cells from the population adhering to the substrate and leaving a suspension of the nonadherent cardiac-derived stem cells.

The invention further provides an isolated nonadherent pluripotent cardiac-derived stem cell, which on proliferation and differentiation produces progeny cells comprising cardiocytes and either chondrocytes or keratinocytes. Some such stem cells on proliferation and differentiation produce progeny cells further comprising at least one cell type selected from the group consisting of an adherent cardiac-derived stem cell, a fibroblast, a smooth muscle cell, and a skeletal muscle cell. Such stem cells can be human or mouse stem cells, for example. Optionally, mouse stem cells can be obtained from a p53 deficient mouse.

The invention further provides an isolated adherent human cardiac-derived stem cell, which proliferates and differentiates to produce progeny cells comprising a cell type selected from the group consisting of a fibroblast, a smooth muscle cell, skeletal muscle cell, a cardiocyte, a chondrocyte, a keratinocyte and an osteoblast.

The invention further provides an isolated adherent cardiac-derived stem cell, which proliferates and differentiates to produce progeny cells comprising a cardiocyte and either a chondrocyte or a keratinocyte.

In another aspect, the invention provides a method of preparing an isolated nonadherent cardiac-derived stem cell. Such a method entails centrifuging a suspension of cells from heart tissue of a subject on a density gradient; isolating a band of cells comprising myocytes; propagating the cells until adherent cardiocytes have died or been discarded leaving suspension cells; and culturing the suspension cells until a population of nonadherent cardiac cells is detectable.

The invention also provides methods of preparing adherent cardiac derived stem cells. Such methods start with a nonadherent cardiac-derived stem cell as described above. The stem cell is the propagated until adherent progeny cells appears. An adherent

cell lacking markers of a cell selected from the group consisting of myoblasts, smooth muscle cells, skeletal muscle cells, cardiocytes, osteoblasts, keratinocytes and chondroblasts, which on proliferation and differentiation produces progeny cells comprising at least one cell type from the group is then identified.

The invention further provides methods of preparing a cardiocyte. Some such methods entail providing a nonadherent cardiac-derived stem cell as described above, propagating the cell under conditions in which the cell proliferates and differentiates to produce progeny cells comprising adherent cells, and identifying an adherent cell with differentiation markers characteristic of a cardiocyte. Alternatively, such methods can start with a nonadherent cardiac-derived stem cell as described above. The cell is propagated under conditions in which the cell proliferates and differentiates to produce adherent progeny cells. An adherent cell is then identified with differentiation markers characteristic of a cardiocyte.

In another aspect the invention provides an isolated population of cells comprising smooth muscle cells, skeletal muscle cells, cardiocytes, fibroblasts, keratinocytes, osteoblasts and chondrocytes.

In another aspect the invention provides methods of treating a patient suffering from necrotic heart tissue. Such methods entail administering to the patient an effective dose of nonadherent or adherent stem cells as described above, whereby the stem cells proliferate and differentiate to produce cardiocytes, which replace the necrotic tissue. In some such methods, the nonadherent stem cells are administered directly to the heart of the patient. In other methods, the nonadherent stem cells are administered intravenously. In some methods, fibroblast growth factor is also administered to the patient to stimulate proliferation and/or differentiation of the nonadherent cells. In some methods, stem cell factor is administered to the patient to stimulate differentiation of the nonadherent cells to cardiocytes. In some methods, the patient has a congestive heart defect. In some methods, the stem cells are obtained from the blood of the patient, and propagated *in vitro* before readministering to the patient.

In another aspect, the invention provides methods of screening potential agents for activity in promoting proliferation and/or differentiation of cardiac-derived stem cells. Such methods entail propagating nonadherent or adherent cardiac-derived stem cell in the presence of a potential agent, and monitoring a change in differentiation state of progeny cells relative to the nonadherent or adherent cardiac-derived stem cells.

In some such methods, the change of differentiation state is adhesion of progeny cells. In some methods, the change in differentiation state is monitored by detecting the appearance of cardiocytes. In some methods, the appearance of adherent cardiac-derived stem cell is monitored.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the differentiation pathways from cardiac tissue-derived stem cells to various differentiated cell types.

Fig. 2 shows a density gradient of cells obtained by digesting heart tissue.

10 The myocyte band is third from top.

DETAILED DESCRIPTION

I. General

The invention provides at least two classes of pluripotent cardiac-derived stem cells. One class of cells is nonadherent and the other is adherent. The adherent cells are partially differentiated progeny of the nonadherent cells. Both classes of stem cells can be propagated and differentiated into a variety of differentiated cell types.

The cardiac-derived stem cells can be isolated from humans and other vertebrate animals. The cells are isolated from a fraction of a suspension of cardiac-derived cells that has typically been discarded as debris by previous workers in the field. A band of myocyte cells is typically isolated from a density gradient, and the cells are propagated in a culture medium on a substrate. Previous workers have retained cells adhering to the substrate and discarded the culture medium in the mistaken belief that it contained only debris. The present inventors have kept the culture medium and discarded the adherent cells. After propagation of the culture medium a population of nonadherent suspension cells becomes apparent.

The cells have several applications in therapy and drug discovery. In therapeutic applications, the cells are administered to patients suffering from heart defect, such as necrotic tissue resulting from a myocardial infarction. The administered stem cells colonize the heart of a patient and give rise to myocardial progeny cells that replace necrotic tissue and/or supplement preexisting heart tissue.

In drug discovery applications, the stem cells are used to screen compounds for activity in promoting or inhibiting differentiation of stem cells to cardiocytes or other mature differentiated cell types. Compounds that promote

differentiation of stem cells to cardiocytes can be used for therapy of patients with heart defects, optionally in conjunction with the stem cells of the invention. Other compounds find uses in other therapeutic application in which promotion or inhibition of stem cell differentiation is desired. Another use for stem cells of the present invention is screening
5 for compounds which expand the stem cell population in culture. Some such compounds also induce differentiation and others do not. Another therapeutic use for cells of the present invention is to provide a method to screen for compounds that promote mobilization of cardiac-derived stem cells from the heart to the circulatory system.

In vivo assays for evaluating cardiac neogenesis include treating neonatal
10 and mature animals with the cells of the present invention. The animals' cardiac function is measured as heart rate, blood pressure, LV pressure-rate production (tdp/dt) and cardiac output to determine left ventricular function. Post-mortem methods for assessing cardiac improvement include: increased cardiac weight, nuclei/cytoplasmic volume, staining of cardiac histology sections to determine proliferating cell nuclear antigen (PCNA) vs.
15 cytoplasmic actin levels (Quaini et al., *Circulation Res.* 75:1050-1063, 1994 and Reiss et al., *Proc. Natl. Acad. Sci.* 93:8630-8635, 1996.)

The cardiac-derived mesenchymal stem cells of the present invention can be used in treatment of disorders associated with heart disease, i.e., myocardial infarction, coronary artery disease, congestive heart failure, hypertrophic cardiomyopathy,
20 myocarditis, congenital heart defects and dilated cardiomyopathy. Cells of the present invention are useful for improving cardiac function, either by inducing cardiac myocyte neogenesis and/or hyperplasia, by inducing coronary collateral formation, or by inducing remodeling of necrotic myocardial area. Cells of the present invention are also be useful for promoting angiogenesis and wound healing following angioplasty or endarterectomy,
25 to develop coronary collateral circulation, for revascularization in the eye, for complications related to poor circulation such as diabetic foot ulcers, for stroke, following coronary reperfusion using pharmacologic methods and other indications where angiogenesis is of benefit.

An ischemic event is the disruption of blood flow to an organ, resulting in
30 necrosis or infarct of the non-perfused region. Ischemia-reperfusion is the interruption of blood flow to an organ, such as the heart or brain, and subsequent restoration (often abrupt) of blood flow. While restoration of blood flow is essential to preserve functional tissue, the reperfusion itself is known to be deleterious. In fact, there is evidence that reperfusion of an ischemic area compromises endothelium-dependent vessel relaxation
35 resulting in vasospasms; and in the heart compromised coronary vasodilation, that is not seen in an ischemic event without reperfusion (Cuevas et al., *Growth Factors* 15:29-40,

1997). Both ischemia and reperfusion are important contributors to tissue necrosis, such as a myocardial infarct or stroke. The cells of the present invention have therapeutic value to reduce damage to the tissues caused by ischemia or ischemia-reperfusion events, particularly in the heart or brain.

- 5 Other therapeutic uses for the present invention include induction of skeletal muscle neogenesis and/or hyperplasia, cartilage regeneration, bone formation, tendon regeneration, neural neogenesis, neogenesis in the pancreas and kidney, and/or for treatment of systemic and pulmonary hypertension. The various specialized cell functions are attained by stimulating the differentiation of cardiac-derived mesenchymal stem cells (MSCs) down the appropriate cell pathway, e.g. into cells of the osteoblast, chondrocyte, skeletal myocyte or fibroblast lineage. The pathway of differentiation is determined by exposure to a growth factor that promotes formation of a particular differentiated cell. Several combinations of growth factor and differentiated cell types are disclosed below and others are known in the art.

- 15 Cardiac-derived MSC-induced coronary collateral development is measured in rabbits, dogs or pigs using models of chronic coronary occlusion (Landau et al., *Amer. Heart J.* 29:924-931, 1995; Sellke et al., *Surgery* 120(2):182-188, 1996 and Lazarous et al., 1996; *ibid.*). Cardiac-derived MSC induced benefits for treatment of stroke are tested *in vivo* in rats utilizing bilateral carotid artery occlusion and measuring histological changes, as well as maze performance (Gage et al., *Neurobiol. Aging* 9:645-655, 1988). Cardiac-derived MSC-induced efficacy in hypertension is tested *in vivo* utilizing spontaneously hypertensive rats (SHR) for systemic hypertension (Marche et al., *Clin. Exp. Pharmacol. Physiol. Suppl.* 1:S114-116, 1995).

25 II. Cells of the Invention

1. Nonadherent Pluripotent Cardiac-Derived Stem Cells

- These cells are characterized by being highly refractory to light, having small size (typically less than 10 microns and often less than 5 microns), round shape and slow growth.

- The cells are further characterized by their capacity to proliferate (i.e., self-renew) in a medium containing a carbon source, a nitrogen source, insulin and transferrin. Addition of stem cell factor (commercially available from Amgen), acid or basic fibroblast growth factor, zFGF-5, leukemia inhibitory factor (commercially available) or increasing concentration of serum stimulates propagation.

The cells are further characterized by capacity to differentiate into several cell types. The cell types include cells in various states of differentiation more advanced

than the nonadherent cardiac-derived stem cells. These cell types include adherent cardiac-derived stem cells (see below), fibroblasts, myoblasts, smooth muscle cells, skeletal muscle cells, cardiocytes, chondroblasts, keratinocytes and chondrocytes.

Probably, adipoblasts, adipocytes, osteoblasts and osteocytes are also present in progeny cells. Tendocytes may also be present. The relationship of the nonadherent cardiac-derived stem cells to differentiated progeny cell lineages and types is shown in Fig. 1.

Cardiac-derived stem cells can be stimulated to differentiate by propagation in FBS-supplemented media. Differentiation can be stimulated by treatment with growth factors, such as stem cell growth factor, and by contact inhibition. Higher concentrations of horse serum, although stimulating proliferation, have a tendency to inhibit differentiation. The type of growth factor used to induce differentiation can bias differentiation toward a selected lineage. Retinoic acid, TGF- β , bone morphogenic proteins (BMP), ascorbic acid, and β -glycerophosphate lead to production of osteoblasts. Indomethacin, IBMX (3-isobutyl-1-methylxanthine), insulin, and triiodothyrocyne (T3) lead to production of adipocytes. aFGF, bFGF, vitamin D3, TNF- β and retinoic acid lead to production of myocytes. zFGF-5 leads to expansion of cardiocytes progenitors although may also be effective later in the pathway from adherent cells to cardiocytes.

(2) Adherent pluripotent cardiac-derived stem cell

Adherent pluripotent cardiac-derived stem cells result from proliferation and partial differentiation of the nonadherent stem cells described in the previous section. The cells are characterized by amorphous shape and lack of differentiation markers tested to-date. Differentiation markers tested include alkaline phosphatase expression, which is a marker for pericytes, osteoblast precursors and chondrocyte precursors; and alpha-actin expression. The cells can be induced to proliferate and/or differentiate into the same cell types as the nonadherent stem cells. The rate of proliferation and the lineage to which differentiation can be induced and can be controlled by supplementation of media with growth factors as described above.

III. Production of Nonadherent Cardiac-Derived Stem Cells

The starting material is a heart or heart biopsy from a human or animal subject. The subject can be embryonic beyond the mesoderm stage, neonatal, infant or adult. If immortalized cells are desired, the starting material can be obtained from the heart of a transgenic animal that is deficient in one or both copies of a tumor suppressor

gene. Examples of tumor suppressor genes include p53, p21, and the retinoblastoma gene. Transgenic mice with a homozygous mutation in p53 are commercially available from Taconic Farms or Jackson Labs.

Methods of processing whole tissue to prepare a suspension of cells are described by e.g., *Methods In Molecular Biology: Animal Cell Culture*, 5, (Pollard et al. eds., Humana Press, NJ, 1990), incorporated by reference). Typically, heart tissue is digested with collagenase, trypsin, other protease and/or DNase to release cells. Polinger, *Exp. Cell Res.* 63:78-82 (1970); Owens et al., *J. Natl. Cancer Instit.*, 53:261-269, 1974; Milo et al., *In Vitro* 16:20-30, 1980; Lasfargues, "Human Mammary Tumors", in Kruse et al. (eds) *Tissue Culture Methods and Applications* (Academic Press, NY, 1973); Paul, *Cell and Tissue Culture*, Churchill Livingstone, Edinburgh, 1975). Intact cells are then separated from debris by low-speed centrifugation. Debris collects in the supernatant. The cell pellet is resuspended and cells are fractionated. Fractionation can be performed on a density gradient such as a Ficoll or sucrose gradient. The cells form four bands as shown in Fig. 2, the myocyte band being the third from the top. The myocyte band is removed and the cells are cultured in a complete medium. Such a medium contains at least a carbon source, a nitrogen source, essential amino acids, vitamins and minerals, and serum. The medium can also be supplemented with growth factors. Cells are grown at about 37°C in an atmosphere of 95% O₂ and 5% CO₂ on a plastic substrate. Initially, most cells are adherent myocytes. However, most of the adherent myocytes (e.g., at least 75 or 90%) die within 5-30 days and small nonadherent cells appear in suspension in increasing concentration. The time period is the shorter end of the above range for p53 deficient cells and the longer end of the range for normal cells. The small nonadherent cells are pluripotent cardiac-derived stem cells.

IV. Differentiation Markers of Different Cell Types

Differentiated cell progeny of stem cells are recognized in part by the presence of differentiation markers. There are three types of muscle cells, cardiocytes (i.e., cardiac muscle cells), striated muscle and smooth muscle cells. The three types of cells derive from a common precursor, termed a myoblast. Cardiac myosin isozyme expression and the cardiac specific pattern of creatine kinase isozyme expression when identified together on the same cell or a clonal population of cells are markers for cardiac muscles cells. Cardiocytes can also be recognized by their bifurcated appearance and capacity to form gap junctions by light microscopy. Such cells can be recognized by

forming an electric potential across confluent cells and detecting transfer of signal across the cells. Muscle α -actin mRNA, and smooth muscle cell actin are differentiation markers of myocytes. Myosin isozyme expression and a muscle-specific pattern of creatine kinase isozyme expression when identified in a cell or clonal population are markers for skeletal muscle cells.

Osteoblast cells secrete bone matrix material. ALP, osteocalcin expression, PTH-induced cAMP expression, and bone mineralization capacity identified together in a cell or clonal population of cells are markers of differentiation for osteoblasts.

Chondrocytes secrete type II cartilage. Aggrecan and collagen Type IIB identified in a cell or clonal population of cells, Alcian Blue staining, which detects production of chondroitin sulfate, are markers for chondrocytes.

Keratinocytes secrete keratin and can be recognized using commercially available stains.

Adipocytes produce lipids and can be recognized by Oil Red O staining.

V. Growth Factors

Basic FGF (also known as FGF-2) is mitogenic *in vitro* for endothelial cells, vascular smooth muscle cells, fibroblasts, and generally for cells of mesoderm or neuroectoderm origin, including cardiac and skeletal myocytes (Gospodarowicz et al., *J. Cell. Biol.* 70:395-405, 1976; Gospodarowicz et al., *J. Cell. Biol.* 89:568-578, 1981 and Kardami, *J. Mol. Cell. Biochem.* 92, 124-134, 1990). Non-proliferative activities associated with acidic and/or basic FGF include: increased endothelial release of tissue plasminogen activator, stimulation of extracellular matrix synthesis, chemotaxis for endothelial cells, induced expression of fetal contractile genes in cardiomyocytes (Parker et al., *J. Clin. Invest.* 85, 507-514, 1990), and enhanced pituitary hormonal responsiveness (Baird et al., *J. Cellular Physiol.* 5, 101-106, 1987.)

zFGF-5 is a type of fibroblast growth factor that is expressed at high levels in human fetal and adult heart tissue. This growth factor is also expressed at decreased levels in fetal lung, skeletal muscle, smooth muscle tissues such as small intestine, colon and trachea. The high level of expression of zFGF-5 in fetal and adult heart and its effects in cardiac tissue-derived cells suggests that ZFGF-5 has particular potency in stimulating proliferation and/or differentiation of cardiac-derived stem cells to cardiocytes. The isolation of zFGF-5 and its properties are described in more detail in

commonly owned PCT US97/18635, filed October 16, 1997 (incorporated by reference). The nucleotide sequence encoding zFGF-5 is described in SEQ ID NO. 1, and its deduced amino acid sequence is described in SEQ ID No. 2. The sequence of zFGF-5 shows some sequence similarity with FGF-8.

5

VI: Immortalization of Cells

Cells that can be continuously cultured and do not die after a limited number of cell generations are termed immortalized. A cell that survives for only 20 to 80 population doublings is considered finite (Freshney, *Culture of Animal Cells* (Wiley-Liss, NY, 1994) herein incorporated by reference), and a cell that survives more than 80, preferably at least 100, cell generations is considered immortalized. Some but not all immortalized cells are tumorigenic. As noted above, cells can be immortalized by using a transgenic animal deficient in a tumor suppressor gene as a donor of heart tissue. Cells from other sources can be immortalized by other means. These means include transformation and expression by a gene whose product plays a role in cell senescence; or overexpression or mutation of one or more oncogenes that override the action of the senescence genes. Expression of genes that result in positive signals for cell proliferation include SV40 large T antigen (Linder et al., *Exp. Cell Res.* 191, 1-7 (1990); polyoma large T antigen (Ogris et al., *Oncogene* 8, 1277-1283, 1993), adenovirus E1A (Braithwaite et al., *J. Virol.* 45, 192-199, 1983), myc oncogene (Khoobyarian et al., *Virus Res.* 30, 113-128, 1993), and the E7 gene of papilloma virus Type 16 (McDougall, *Curr. Top. Microbiol. Immunol.* 186:101-119, 1994).

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VII: Therapeutic Regimes

Heart disease is the major cause of death in the United States, accounting for up to 30% of all deaths. More than 5 million people are diagnosed with coronary disease in the US. Coronary disease can be due to congestive heart failure, hypertrophic cardiomyopathy, cardiomyopathy, viral infection or myocardial infarction (MI). Myocardial infarction accounts for 750,000 hospital admissions per year in the US. In MI patients, ischemia stimulates growth of fibroblasts and promotes development of greater than normal quantities of fibrous tissue to replace necrotic muscular tissue. Risk factors for MI include diabetes mellitus, hypertension, truncal obesity, smoking, high levels of low density lipoprotein in the plasma or genetic predisposition.

30

Although some proliferation of cardiocytes apparently occurs with normal aging humans and animals (Olivetti et al., *J. Am. Coll. Cardiol.* 24(1), 140-9 (1994) and Anversa et al., *Circ. Res.* 67, 871-885, 1990), this is inadequate to repair damage due to coronary disease. Thus, necrosis that occurs in MI and other coronary diseases is essentially irreversible.

Such conditions are treated by administration of cardiac-derived stem cells as described above. Either adherent or nonadherent cells can be used. The cells can be administered either intravenously, intracoronary or intraventricularly. A catheter can be used for the latter two routes of administration, which are more usual for adherent cells.

Cells are administered in a therapeutically effective dosage. Such a dosage is sufficient to generate significant numbers of new cardiocytes cells in the heart, and/or at least partially replace necrotic heart tissue, and/or produce a clinically significant change in heart function. A clinically significant improvement in heart performance can be determined by measuring the left ventricular ejection fraction, prior to, and after administration of cells, and determining at least a 5% increase, preferably 10% or more, in the total ejection fraction. Standard procedures are available to determine ejection fraction, as measured by blood ejected per beat. Dosages can vary from about $100-10^7$, $1000-10^6$ or 10^4-10^5 cells.

Cells can be administered as pharmaceutical compositions, which can also include, depending on the formulation desired, pharmaceutically-acceptable, typically sterile, non-toxic carriers or diluents, which are defined as vehicles commonly used to formulate pharmaceutical compositions for animal or human administration. The diluent is selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological phosphate-buffered saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like.

Administration of stem cells can be preceded, accompanied or followed by administration of growth factor(s) that stimulate proliferation and/or differentiation of the stem cells into cardiocytes. Growth factors can be administered intravenously or intraventricularly. Growth factors are administered in a dosage sufficient to cooperate with administered stem cells in generating significant numbers of new cardiocytes cells in the heart, and/or at least partially replace necrotic heart tissue, and/or produce a clinically significant change in heart function.

Suitable growth factors include zFGF-5 and stem cell growth factor.

In some methods, cardiac-derived stem cells are administered in combination with anti-inflammatory agents that arrest, reverse or partially ameliorate inflammation associated with coronary disease. Suitable antiinflammatory agents include antibodies to Mac-1, and L-, E and P-selectin (see Springer, *Nature* 346, 425-433 (1990).

- 5 Osborn, *Cell* 62, 3 (1990); Hynes, *Cell* 69, 11 (1992)). Cardiac-derived stem cells can also be administered with diuretics, ACE inhibitors and β -adrenergic blockers.

In some methods, the recipient patient of stem cells and the donor from which the cells are obtained are HLA-matched to reduce allotypic rejections. In other methods, cells are administered under cover of an immunosuppressive regime to reduce the risk of rejection. Immunosuppressive agents that can be used include cyclosporin, corticosteroids, and OKT3. In other methods, immune responses are avoided by obtaining stem cells from the patient that is to be treated. Stem cells can be obtained by biopsy of heart tissue, and expanded *in vitro* before readministration. Alternatively, given the present provision of isolated cardiac-derived stem cells, differentiation markers can be identified for these cells, and the cells can be isolated from the blood of the patient to be treated.

VIII. Use of Cardiac-Derived Stem Cells in Drug Screening

The cardiac-derived stem cells described above can be used to test compounds for activity in promoting or inhibiting proliferation and/or differentiation of the cells. In general, a compound being tested is contacted with a population of cardiac-derived stem cells, optionally, in the presence of other agents known to promote or inhibit the metabolic pathway or phenotype of interest, and phenotypic and metabolic changes are monitored in comparison with a control in which the compound being tested is absent. Compounds to be tested include known or suspected growth factors, and analogs thereof, libraries of natural compounds not previously known to have activity in promoting proliferation or differentiation and combinatorial libraries of compounds. Large combinatorial libraries of the compounds can be constructed by the encoded synthetic libraries (ESL) method described in Affymax, WO 95/12608, Affymax, WO 93/06121, Columbia University, WO 94/08051, Pharmacoopia, WO 95/35503 and Scripps, WO 95/30642 (each of which is incorporated by reference for all purposes). Peptide libraries can also be generated by phage display methods. See, e.g., Devlin, WO 91/18980.

Compounds that cause cardiac-derived stem cells to proliferate and/or differentiate into cardiocytes are useful as therapeutic agents in the same conditions as the

- cardiac-derived stem cells are useful. Such compounds can be administered alone to stimulate proliferation and differentiation of endogenous cardiac-derived stem cells, or can be administered in conjunction with exogenous cardiac-derived stem cells. Such compounds are screened for proliferating activity by contacting them with cardiac-
- 5 derived stem cells in growth media, and monitoring an increase in cell count, or incorporation of ^3H -thymidine. Compounds are screened for promoting differentiation to cardiocytes by monitoring cells with the characteristic morphological appearance and differentiation markers of cardiocytes as note above.
- 10 Similarly, compounds can be monitored for activity in promoting differentiation of cardiac-derived stem cells to other differentiated cell types, such as smooth muscle cells, skeletal muscle cells, osteoblasts and chondrocytes. Activity is detected by detecting the characteristic morphological appearance and differentiation markers of one of these cell types. Compounds with activity in promoting differentiation to one of the above cell types are useful in treating patients with degenerative diseases of
- 15 bone, muscle or cartilage.

Compounds that inhibit differentiation of stem cells to certain cell types such as adipocytes can also be useful in some circumstances. For example, compounds that inhibit differentiation of stem cells to adipocytes can be used in treating obesity. Such compounds are identified by contacting a compound under test with cardiac-derived

20 stem cells under conditions that would otherwise lead to differentiation of the stem cells to a certain cell type, and monitoring a decreased frequency or extent of conversion to the cell type relative to a control in which the compound is omitted.

- Compounds identified as therapeutic agents by such screening with the cell lines of the invention are formulated for therapeutic use as pharmaceutical compositions.
- 25 The compositions can also include, depending on the formulation desired, pharmaceutically-acceptable, non-toxic carriers or diluents, as described above.

EXAMPLES

1. Materials

30 a. Serum-free Media for Myocytes

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (1:1; Gibco Laboratories)

Insulin (JRH 5C2059), 1 $\mu\text{g}/\text{ml}$

Transferrin (JRH 6K2222), 5 $\mu\text{g}/\text{ml}$

Selenium (Aldrich Chem. Co. 20010/7), 1 nM

Thyroxine (Sigma T-0397), 1 nM

Ascorbic Acid (Sigma A-4034), 25 µg/ml

LiCl (Sigma L-0505), 1 nM

5

b. Plating Media (PM)

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12

(1:1; Gibco Laboratories), contain 15% HIA FBS

10 **c. Media for Myocyte Isolation**

Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (1:1; Gibco Laboratories)

DF 20, contains 20% HIA FBS

DF 10, contains 10% HIA FBS

15 **DF 5, contains 5% HIA FBS**

HIA FBS (HyClone lot# AEL4614)

d. Enzymes for Digestion of Heart Tissue

Collagenase (Worthington LS004176), 2% solution

20 **DNAse (Worthington LS002007), 0.5% solution**

Enzymes are dissolved in PBS (Gibco Laboratories) w/ 1% Glucose

e. Percoll Purification

Percoll (Pharmacia #17-0891-01)

25 **Add buffers (g/L for 1x; or g/100ml for 10x), pH 7**

6.8 NaCl

1.0 Dextrose

1.5 NaH₂PO₄

0.4 KCL

30 **0.1 MgSO₄**

4.76 HEPES

0.02 Phenol Red - Sigma

All reagents were passed through a 0.22 µM filter before use.

A stock solution was made using 9 parts (45 ml) Percoll, plus 1 part (5 ml) 10x Ads without Phenol Red.

Gradient steps were made as follows.

5

Density (g/ml)	Percoll Stock (ml)	1 x Ads (\pm Phenol Red) (ml)
1.050	18 ml	32 ml without Phenol Red
1.060	22.2 ml	27.8 ml with Phenol Red
1.082	31.6 ml	18.4 ml without Phenol Red

f. Human Fibronectin Plates

Stock solution is 1 mg/ml (1000 μ g/ml)

Coating concentration 1 μ g/cm²

- 10 Dilute stock solution to 10 μ g/ml (100X: 200 μ l in 20 ml SFM)

Plate 3 ml/60 mm tissue culture dishes

Incubate at room temperature for 1 hour

Aspirate remaining material

- 15 Rinse plates carefully with dH₂O - avoid scraping bottom surface.

Plates are ready for use, store at 4°C

2. Isolation of Nonadherent Cardiac-Derived Stem Cells from P53 Deficient Mice

In this example, a code is used to designate months. Thus, A, B and C represent successive months starting with A.

20

On A/19, hearts from 10 P53^{-/-} female mice (about 2 months of age) were digested as follows:

- 1) The hearts were minced with scissors in a minimal volume of PBS(-).
- 2) PBS(-) + 1% glucose was added for a final volume of 18 ml.
- 3) 1 ml of DNase and 1 ml collagenase were added.
- 4) The suspension was agitated in a 50 ml Falcon tube on a rotary shaker at 37°C
5 for 30 minutes.
- 5) This first supernatant was discarded as it contains mostly RBC debris and fibroblasts.
- 6) Steps 2-5 were repeated until the hearts were completely digested.
- 7) After each 30 min agitation period (except for the first digestion, which was
10 discarded), the supernatant was removed and added to 20 ml of DF20. The supernatant with the DF20 was spun at 1650 rpm, 4°C for 10 min. The resulting pellet was saved and resuspended in 10 ml DF10 and kept on ice.
- 8) After complete digestion of the hearts, the pellets were combined (5 pellets per
15 50 ml Falcon tube) and spun at 1650 rpms, 4°C for 10 min.
- 9) The final resulting pellet was resuspended in 1.082 gm/ml Percoll (12 ml).
- 10) A Percoll gradient was prepared in a 50 ml Falcon tube as follows:
12 ml 1.050 gm/ml Percoll loaded, then 12 ml 1.060 gm/ml Percoll loaded
from the bottom, followed by the cell suspension in 1.082 gm/ml Percoll,
loaded from the bottom. The 50 ml Falcon tube was spun in a Beckman
20 CS-6R centrifuge for 30 min at 3000 rpm, RT.
- 11) The resulting myocyte fraction in the band between the lower 1.082 gm/ml Percoll
and the middle 1.06 gm/ml Percoll layers was removed and an equal volume of
DF10 was added.
- 12) The myocyte fraction was spun at 1650 rpm for 10 min at RT.
- 25 13) The resulting pellet contained 3.75×10^6 cells, which were plated equally into 5
wells in 5 ml DF5.
- 14) On A/20 the media was changed to plating media (PM) in 6-well tissue culture
plates, which was replaced again with fresh PM on A/22 and A/25. Adherent
30 beating cardiocytes were apparent from A/20 through A/25, although in
diminishing numbers. Additionally, very small suspension cells were present in
the wells.
- 15) On A/26 these suspension cells were split 1:3 into PM, PM plus 1% horse serum
(HS), and PM plus 10% HS (HyClone, Logan, Utah).

16) On B/01 the suspension cells in PM plus 1% horse serum (HS) and PM plus 10% HS were split 1:10 into T25 flasks (0.5 ml cell suspension plus 4.5 ml fresh PM with either 1% HS or 10% HS). On B/15 the suspension cells in PM plus 1% HS were split 1:1. The flasks were not manipulated again until C/19. The flasks were
5 incubated at 37°C in 95% O₂ and 5% CO₂.

On C/19, the cells maintained in PM plus 10% HS were still viable by light refraction and trypan blue exclusion. Cells in PM only were not viable. Cells in PM and 1% HS had become adherent cells and are described in more detail below. The suspension cell density was determined, using a hemacytometer, to be 6×10^6 /ml.

10 Also, on C/19, the cells grown in PM + 10% HS were spun at 1500 rpm for 10 min and resuspended at 0.5×10^6 /ml in serum free media (SFM), SFM plus 1% HS, SFM plus 10% HS, PM, PM plus 1% HS, or PM plus 10% HS.

On C/26 the suspension cells in PM plus 10% HS were at 8.5×10^5 /ml, while the suspension cells in SFM plus 10% HS were at 8.75×10^5 /ml. Half of these
15 suspension cells (both the SFM and PM cells) were resuspended in PM plus 10% HS or SFM plus 10% HS, and were plated on methylcellulose in the presence of various cytokines and growth factors. The suspension cells did not form colonies on methylcellulose, even after 12 days in culture suggesting that the cells could be primitive stem cells.

20 At this time, culturing the suspension cells in 10% HS no longer appeared to impact the cells significantly, so the cells were passaged in PM only or SFM only. zFGF-5 (150 pg/ml) enhanced proliferation of the cells 2.5-5.0 fold, and stem cell factor (SCF 10 ng/ml) enhanced proliferation about 1.0-2.0 fold determined by short-pulse tritiated-thymidine uptake.

25 A one month treatment of the suspension cells with 10 ng/ml SCF in PM resulted in the appearance of an adherent layer of cells of mixed lineage designated SCF/PM-derived cells. By contrast, treating the suspension cells with SCF in SFM did not give rise to adherent cell lineages, implying that some other growth factor present in the 15% FBS found in PM is essential, in addition to the SCF. Addition of zFGF-5
30 (150 pg/ml) to the flasks with SCF in PM prevented the induction of the adherent cell lineages, suggesting that zFGF-5 blocked the differentiating activity of the SCF.

By light microscopy, the morphology of the SCF/PM-derived cells suggested lineages which include cardiocytes, chondrocytes, fibroblasts, smooth muscle myocytes, and skeletal muscle myoblasts. Cardiocyte-like cells had the appearance of

cigars or driftwood. Chondrocyte-like cells had the appearance of nests or cobblestone streets. Fibroblasts were long and spindly. Cells with a phenotype of smooth muscle myocytes were amorphous or star-shaped, skeletal muscle cells had the shape of a pen. Myoblasts were intermediate between fibroblasts and skeletal muscle cells. Other lineages are probably present, and can be characterized by using immunohistochemistry, immunofluorescence and FACS analysis.

3. Adherent Cardiac-Derived Stem Cells

On C/19, the cells maintained in PM plus 1% HS had differentiated into adherent cells of mixed morphology. These cells were trypsinized, pelleted, and then split 1:6 into SFM, SFM plus 1% HS, SFM plus 10% HS, PM, PM plus 1% HS or PM plus 10% HS.

On C/26, the adherent cells were expanded in PM only as the different media or HS concentrations did not have any noticeable affect on the adherent cell morphology. By light microscopy the adherent cells appear to be a mixed population including pericytes, fibroblasts, cardiocytes, smooth muscle myocytes, skeletal muscle myocytes.

The adherent cells were subcloned on C/08. From the subcloning, 11 clones were obtained and expanded on plating media in 95% O₂/ 5% CO₂ at 37°C. The cells were characterized by the following criteria.

- (1) Morphology by light microscopy.
- (2) Alkaline phosphatase staining (identifies pericytes, osteoblast precursors and chondrocyte precursors and mature osteoblasts and chondrocytes).
- (3) Acetylated LDL uptake (endothelial cell marker).
- (4) MF-20 Ab (Mouse anti-adult chicken pectoralis myosin, which crossreacts with mouse myosin and detects both adult skeletal and cardiac myocyte myosin).
- (5) M0636 (Dako #5/56 mouse anti-human muscle actin; recognizes alpha actin in cardiac, skeletal and smooth muscle myocytes, and gamma actin in smooth muscle myocytes).
- (6) Mitogenic response to zFGF-5, acidic FGF and basic FGF. This test tests for proliferation capacity. The cells were plated in 96-well plates in PM and grown to confluence and then switched to serum-free media for 24 hr after which growth factor and tritiated thymidine was added.

- (7) Production of zFGF-5 mRNA identified by Northern blotting which is expressed at high concentration by cardiocyte lineage cells.
- (8) Differentiation induced by contact-inhibition or passage in SFM to identify proliferation and/or differentiation capacity.
- 5 (9) Differentiation induced by ascorbic acid/ α -glycerophosphate (AA/ α -GP) to identify osteoblasts.

(a) Mixed population propagated in PM + 1% HS before isolating clones

10 Cells in this population showed different morphologies characteristic of pericytes, fibroblasts, cardiocytes, smooth muscle myocytes, skeletal muscle myocytes, and other cells.

About 20-25% of cells were positive for alkaline phosphatase indicating cells of osteoblast or chondrocyte lineage. All cells were negative for acetylated LDL uptake indicating an absence of endothelial cells. All cells were negative for MF-20
15 binding indicating an absence of terminally differentiated skeletal cells. All cells were positive for M0636 binding indicating the presence of precursors of cardiocytes, myocytes, skeletal muscle cells and smooth muscle cells.

The cells showed a mitogenic response to zFGF-5 (cFGF), acidic FGF (aFGF) and basic FGF. bFGF was most potent in inducing a mitogenic response. The
20 response increased in a concentration dependent manner from 1 pg/ml up to 1 μ g/ml.

On treatment with serum free media, the cells showed moderate growth and differentiated into cells with an appearance akin to frying eggs. This appearance suggests early stage myocytes.

On treatment with ascorbic acid/ β -GP, cells differentiated into
25 chondrocyte-like cells by day 6 by morphology. No mineralization occurred even after Day 25 indicating absence of osteoblast lineage cells.

Clone 1D9:

Clone 1D9 was isolated from a single cell of the mixed population
30 described above in 3a. Some cells propagated from 1D9 showed morphology of cardiocytes, and other cells showed different morphology. A few cells stained faintly positive for alkaline phosphatase. The cells did not take up acetylated LDL. MF-20 did not bind to the cells. M0636 did bind to cells indicating that some cells were of smooth muscle, skeletal muscle, and/or cardiocyte lineage. zFGF-5, acidic FGF and basic FGF

- all induced a mitogenic response and bFGF was most potent. Serum-free media induced slow growth but not obvious morphological changes. Contact-dependent differentiation produced chondrocytes, cardiocytes, and other cell types recognized by morphology. Ascorbic acid/ β -GP-treatment resulted in appearance of chondrocyte-like cells on Day 6.
- 5 No mineralization occurred even after Day 25. These results indicate that clone 1D9 gives rise to differentiated cells of different lineages.

Clone 2E7:

- Cells expanded from 2E7 have the morphology of myoblast (skeletal myocyte/fibroblast/adipocyte precursor).
- 10 The cells were negative for alkaline phosphatase staining, acetylated LDL uptake and MF-20 binding. The cells were positive for M0636 binding indicating the presence of precursors of smooth, skeletal and/or cardiocyte muscle cells. The cells proliferated in response to zFGF-5, acidic FGF and basic FGF, with basic FGF being the
- 15 most potent. On treatment with serum free media, the cells proliferated and changed morphology to appear as large polygonal cells, which may be early stage myocytes. The cells were negative for MF-20 binding. On treatment with ascorbic acid/ β -GP, cells differentiated to "ridge-like" formations on Day 11. No mineralization was observed even after day 25.

- 20 **Clone 1G11:** Clone 1G11 was also isolated as a single cell from the mixed population of adherent p53 deficient cells described above. Cells expanded from 1G11 had the morphology of skeletal myocytes, smooth muscle myocytes, cardiocytes, and other cell
- 25 types. About 20-25% of cells stained positive for alkaline phosphatase. The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF, with bFGF the most potent and bFGF and aFGF equally active. Addition of serum free media caused a slow growth rate and the appearance of star-shaped cells, which are myocyte precursors. The cells did not bind MF-20. Treatment with ascorbic acid/ β -GP did not result in mineralization even
- 30 after Day 25.

Clone 2B6:

This clone showed the morphology of skeletal myocytes, smooth muscle myocytes, cardiocytes, and other cell types. Cells were negative for alkaline phosphatase

staining and acetylated LDL uptake, MF-20 binding. The cells showed a mitogenic response to bFGF and aFGF, but not to zFGF-5. SFM-induced differentiation, and slower growth rate, producing thicker, rod-shaped cells. Treatment with ascorbic acid/ β -GP induced resulted in cells gathering together in 'ridge-like' formations on Day 11. No mineralization occurred even after Day 25.

Clone 2A7:

This clone showed the morphology of myoblasts (skeletal myocyte/fibroblast/adipocyte precursors). The cells were negative for alkaline phosphatase staining, acetylated LDL uptake, MF-20 binding. The cells were slightly positive for M0636 binding. The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF. aFGF and bFGF were most potent, all 3 FGFs were equally active. SFM caused no change in growth rate or morphology. Ascorbic acid/ β -GP treatment did not result in mineralization even after Day 25.

Clone 2G7:

This clone had the morphology of multinucleated cardiocytes, and other cell types. Some cells were positive for alkaline phosphatase staining. The cells were negative for acetylated LDL uptake, and MF-20 binding. The cells were positive for M0636 binding. The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF, with bFGF being most potent, and aFGF and bFGF being equally active. SFM induced moderate growth rate, and no distinct morphology change. Cells were negative for MF-20 binding. Ascorbic acid/ β -GP treatment did not result in mineralization even after Day 25.

Clone 2F11:

This clone had the morphology of myoblasts (skeletal myocyte/fibroblast/adipocyte precursors). 5% of cells stained with alkaline phosphatase. The cells were negative for acetylated LDL uptake, MF-20 binding, and M0636 binding. SFM induced moderate growth rate resulting in star-shaped cells. Ascorbic acid/ β -GP-induced differentiation resulted in cells gathering in a 'ridge-like' formation on Day 11.

No mineralization occurred even after Day 25.

Clone 2A6:

These cells had a stellate appearance characteristic of myoblasts. The cells were negative for alkaline phosphatase staining, negative for acetylated LDL, negative for MF-20 binding and negative for M0636 binding. The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF, with bFGF being the most potent, and all 3 FGFs being equally active. SFM treatment caused moderate growth and the appearance of some large star-shaped cells. Treatment with ascorbic acid/ β -GP induced differentiation with cells gathering together in 'ridge-like' formations on Day 11 (many formations were evident). No mineralization occurred even after Day 25.

Clone 2G12:

These cells showed the morphology of cardiocytes, skeletal myocytes, smooth muscle myocytes, and others. The cells were negative for alkaline phosphatase staining, acetylated LDL uptake, MP-20 binding, M0636 binding.

The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF. bFGF and cFGF were equally potent and active from 10 fg/ml up to 1 μ g/ml. FGF stimulation regenerated the original suspension cell progenitor population and produced zFGF-5 mRNA. zFGF-5 150 pg/ml (1 day) stimulation produced clear adult cardiocytes by morphology. Treatment for five days resulted in formation of haystack structure, ridges and troughs. SFM treatment resulted in strong growth, loss of multinucleation, and appearance of large polygonal cells. Contact-dependent differentiation produced cardiocytes, chondrocytes, and other cell types. Ascorbic acid/ β -GP-treatment resulted in differentiation and larger cells by Day 6. No mineralization occurred even after Day 25.

Clone 2D6:

The cells had a stellate or haystack appearance when sub-confluent, suggesting myoblasts (skeletal myocyte/fibroblast/adipocyte precursor). The cells were negative for acetylated LDL uptake, negative for alkaline phosphatase staining, negative for MF-20 binding and positive for M0636 binding. SFM treatment caused rapid growth, and loss of haystack or stellate appearance. Ascorbic acid/ β -GP treatment resulted in appearance of chondrocyte-like cells on Day 6. No mineralization occurred even after Day 25.

Clone 2H6:

The cells showed the morphology of cardiocytes and other cells. The cells were negative for alkaline phosphatase staining, acetylated LDL uptake, MF-20 binding and positive for M0636 binding. The cells showed a mitogenic response to zFGF-5, acidic FGF and basic FGF. bFGF was the most potent, and all 3 FGFs were equally active. SFM treatment caused reduced growth rate, reduced adherence to plastic, and appearance of large polygonal cells. Treatment with ascorbic acid/ β -GM resulted in cells gathering together in 'ridge-like' formations on Day 11. No mineralization occurred even after Day 25.

5. Isolation of Cardiac-Derived Stem Cells from Neonatal Mice

- 1) 1-4 day old neonates (mice) are sacrificed by cervical dislocation and placed in a 70% ETOH bath. When 10-15 neonates have been collected, one is placed on its back and a midline sternotomy is performed. Pressure is applied to the chest cavity with forceps, the heart popped out and the ventricle easily excised. The ventricles are placed in ice cold PBS (50 ml Falcon Tube, with PBS, on ice), with approximately 100 ventricles in each tube.
- 2) When all the hearts have been collected, rinse in PBS several times. Then heart tissue is minced with scissors in a minimal volume of PBS, and rinsed again in PBS several times. (Rinse until all red blood cells and debris is removed). From step 3) PBS is supplemented with 1% Glucose.
- 3) a) To begin dissociation, hearts are brought up in 18 ml PBS.
b) 1 ml of each stock solution of DNase/Collagenase is added.
- 4) Agitate on a rotary shaker at 37°C for 30 minutes. (1. DIGESTION)
- 5) Discard supernatant from the 1. Digestion, which contains mainly fibroblasts, red blood cells and debris.
- 6) a) To begin dissociation, hearts are brought up in 18 ml PBS.
b) 1 ml of each stock solution of DNase/Collagenase is removed.
- 7) Agitate on a rotary shaker at 37°C for 20 minutes.
- 8) Transfer supernatant (20 ml) from tube, to a 50 ml Falcon Tube containing 20 ml DF20.

Mix carefully (turn tube upside-down).

- 9) Spin tube(s) at 1650 rpm, 4°C for 10 min.

- 10) Keep pelleted cells on the bottom, and discard supernatant (~40 ml).
- 11) Add 10 ml of DF10 to pellet, mix well up and down (using the sterile pipette), keeping cell suspension(s) on ice.
- 5 Repeat steps 6)-11) until all tissue is digested.
- 12) When dissociation is complete, combine all steps 11).
- 13) Pellet cells (spin tubes at 1650 rpm 4°C for 10 minutes, discard supernatant).
- 14) Resuspend myocytes in 1.082 g/ml Percoll (12 ml Percoll/100 neonates).
- 10 15) Prepare Percoll Gradient using 10 ml pipette to load from bottom 12 ml each of 1.050 g/ml, 1.060 g/ml, then 1.082 g/ml (containing cells) Percoll in as many 50 ml Falcon tubes as needed. (1 tube Percoll Gradient/100 neonates).
- 16) Centrifuge for 30 minutes at 3000 rpm, room temperature. A Beckman CS-6R centrifuge is suitable.
- 15 18) After the centrifugation step in the Percoll Gradient, 4 bands appear (see Fig. 2).
- 19) Aspirate off the upper band, collect (fibroblast layer first, if want to keep, transfer into a 50 ml Falcon Tube, add same amount of DF10 volume cell suspension collected), then collect myocyte layer, transfer into a 50 ml Falcon Tube, add same amount of DF10 as volume cell suspension collected.
- 20 20) Mix well but carefully, and spin cells at 1650 rpm, 10 min, room temperature.
- 21) Discard supernatant. Add 5 ml DF5/"100 neonates".
- 22) Incubate myocytes on human fibronectin (HFN) for 1 hr at 37°C. The remaining fibroblasts will attach to coated plate within an hour.
- 23) Collect non-adherent cells, wash the dish several times with DF5 to make sure that
- 25 all nonadherent cells are collected.
- 24) Spin cells 10 min at 1650 rpm, remove supernatant, and resuspend cells in DF5.

6. Expansion of MSCs from Bone Marrow

- Assays were performed to measure the frequency of fibroblast colony forming units from monkey low density, non-adherent cells isolated from bone marrow. This assay is indicative of mesenchymal stem cell frequency.

One half of a 96 well microtiter plate is inoculated with cells at a density of 10,000 cells/well and the other half of the plate is inoculated with cells at a density of

1,000 cells/well. The culture medium is α MEM (GIBCO-BRL, Gaithersburg, MD), 2% bovine serum albumin, 10 μ g/ml insulin, 200 μ g/ml transferrin, antibiotic and 50 μ M β -Mercaptoethanol. The cells are incubated at 37°C in 5% CO₂ for 14 days and then stained with toluidine blue to improve cell visibility and examined microscopically. Positive wells have at least 50 cells exhibiting a "stromal" morphology, i.e., large, spread out cells. The positive control is medium containing 20% fetal bovine serum. Results demonstrated that zFGF5, at a concentration of 100 ng/ml increased the frequency of CFU-F to levels equivalent to the positive control of 20% FBS.

10 7. Identification of Cardiac-Derived MSCs for zFGF5

Identification of a putative mesenchymal stem cell as a target (i.e. having a receptor) for zFGF5 was made using FITC-labeled protein and either neonatal mouse or fetal lamb (third trimester) heart tissue.

zFGF5, purified as described above, was dialyzed into 0.1-M sodium bicarbonate pH 9.0. Fluorescein isothiocyanate (FITC; Molecular Probes, Eugene, OR) was dissolved at 1 mg/ml in the same buffer without exposure to strong light. The mixture was prepared containing 1 mg FITC/1 mg zFGF5, and reacted for 1-2 hours in the dark at room temperature. The reaction was stopped by adding 1 M glycine to a final concentration of 0.1 M, then reacted for 1 hour at room temperature. The mixture was then dialyzed against 0.1 M sodium bicarbonate to make a 1:500-1:1000 dilution for 3 hours. The dialysis solution was changed and the process repeated for 3-18 hours to remove unlabeled FITC.

Neonatal mouse or fetal lamb heart ventricles were isolated, minced, and repeatedly washed in phosphate buffered solution (PBS) until all red blood cells and debris were removed. The minced ventricles were placed in a solution containing 18 ml PBS and 1% glucose and 1 ml of 2% DNase/Collagenase solution was added. The mixture was incubated on a shaker for 30 minutes at 37°C. The supernatant was discarded and the process was repeated once more. After incubation, the supernatant (~20 ml) was transferred to a tube containing 20 ml DF 20 (Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12, 1:1 (GIBCO-BRL, Gaithersburg, MD) and 20% fetal bovine serum). After mixing, the tubes were centrifuged at 1650 rpms in a Beckman CS-6R centrifuge (Beckman, Fullerton, CA) at 4°C for 10 minutes. The supernatant was discarded and the pellet was resuspended in DF 10 (10% FBS). The cells were kept cold and spun again and resuspended in 40 ml of DF 10. The cell mixture was passed over a 40 μ m filter (Becton Dickinson, Detroit, MI) and counted using a hemacytometer.

The cells were incubated in FITC-labeled zFGF5 at 4°C for 30 minutes at a concentration of 2×10^6 cells/1 μ g zFGF5. After incubation, the cells were spun at

1650 rpms in a Beckman CS-6R centrifuge (Beckman) for 5 minutes. The supernatant was discarded and the pellet washed once in 10 ml of DF 10 and resuspended in 4 ml DF 10.

- 10 μ l of MACS anti-FITC microbeads (Miltényi Biotech, Auburn, CA) were mixed with 10^7 cells in 4 ml of DF10 and incubated at 4°C for 30 minutes.

- MACS positive selection type LS+ separation columns (Miltényi Biotech) were washed with 3 ml of MAC buffer (PBS, 0.5% BSA, 2 mM EDTA) and the cell/bead mixture was washed in 10 ml MAC buffer and then resuspended in 6 ml MAC buffer. The cell/bead mixture was divided between the two columns and the first negative fraction was discarded. 1.5 ml of 0.6 M NaCl was added to each column and eluted but not collected. The columns were then washed with 1.5 ml MAC buffer. The cells bound with FITC-labeled zFGF5 were collected by adding 3 ml MAC buffer, removing the column from the magnet and flushing out the positive cells using the plunger. The positive cell fraction was plated in a T75 flask and 50 ml of plating medium was added (DF with 15% FBS and antibiotics). The cells were incubated at 37°C for 1 week and counted. The yield of positive cells was approximately 0.1% of original total cells counted.

- Cells binding FITC-labeled zFGF5 were examined by transmission electronmicroscopy (TEM). The cells were between 3-5 microns in diameter. The cell nuclei occupied the majority of the cell volume, and few cytoplasmic organelles were apparent. The phenotype identified by TEM identifies the zFGF5-isolated cells as primitive mesenchymal stem cells.

8. In vivo Study of Cardiomyopathic Rats

- 25 Rats infused subcutaneously with epinephrine for 2 weeks develop a cardiomyopathy quite similar to human idiopathic dilated cardiomyopathy (Deisher et al., *Am. J. Cardiovasc. Pathol.* 5(1):79-88, 1994 and Deisher et al., *J. Pharmacol. Exp. Ther.* 266(1):262-269, 1993.)

- The effect of cardiac-derived MSCs on the initiation and progression of the catecholamine-induced cardiomyopathy is evaluated by administering cardiac-derived MSCs isolated from healthy rats with a similar genetic background by intra-pericardial, intra-coronary, intra-arterial, intra-ventricular or intra-venous injection to rats receiving subcutaneous infusions of epinephrine or saline.

- In one protocol, rats (300 gm) are implanted with epinephrine in an osmotic pump and are injected with cardiac-derived MSCs or control cells and mortality is monitored for 2 weeks, at the end of which the rats are sacrificed, the hearts are

weighed wet, and fixed in 10% neutral buffered formalin for histology. Prior to sacrificing, cardiac function is measured.

Measurement include body weight, heart weight, cardiac fibrosis and cardiohistomorphometry in epinephrine-infused rats. Cardiac fibrosis is determined by scoring Masson's Trichrome stained heart sections. Three sections are scored for each heart, and the average score taken. Positive results indicate that infusion of cardiac-derived MSCs can be beneficial in the setting of heart failure of varying etiologies, of which can include myocardial infarct (MI), idiopathic dilated cardiomyopathy (IDCM), hypertrophic cardiomyopathy, viral myocarditis, congenital abnormalities, and

10 obstructive diseases.

It is apparent from the foregoing that the invention includes a number of uses, some of which can be expressed concisely as follows: The invention provides for the use of nonadherent cardiac-derived stem or adherent cardiac-derived stem cells in the treatment of disease and or in the discovery of drugs for use in the same. The invention further provides for the use of nonadherent or adherent cardiac-derived stem cells in the manufacture of a medicament for treatment of disease.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

WHAT IS CLAIMED IS:

- 1 1. An isolated nonadherent pluripotent cardiac-derived human stem
2 cell, which on proliferation and differentiation produces progeny cells comprising a cell
3 type selected from the group consisting of an adherent cardiac-derived stem cell, a
4 fibroblast, a smooth muscle cell, a skeletal muscle cell, a cardiocyte, a keratinocyte, an
5 osteoblast and a chondrocyte.
- 1 2. The isolated nonadherent cardiac-derived stem cell of claim 1,
2 which on differentiation produces progeny cells including at least two cells selected from
3 the group.
- 1 3. The isolated nonadherent cardiac-derived stem cell of claim 1,
2 which on proliferation and differentiation produces progeny cell comprising all of the cell
3 types selected from the group.
- 1 4. The isolated nonadherent cardiac-derived stem cell of claim 1 that
2 is immortalized.
- 1 5. The isolated nonadherent cardiac-derived stem cell of claim 1
2 produced by
3 propagating a population of heart tissue-derived cells in a liquid medium
4 on a substrate;
5 discarding cells from the population adhering to the substrate and leaving a
6 suspension of the nonadherent cardiac-derived stem cells.
- 1 6. An isolated nonadherent pluripotent cardiac-derived stem cell,
2 which on proliferation and differentiation produces progeny cells comprising cardiocytes
3 and either chondrocytes or keratinocytes.
- 1 7. The isolated nonadherent pluripotent cardiac-derived stem cell of
2 claim 6, which on proliferation and differentiation produces progeny cells further
3 comprising at least one cell type selected from the group consisting of an adherent
4 cardiac-derived stem cell, a fibroblast, a smooth muscle cell, and a skeletal muscle cell.

1 8. The isolated nonadherent cardiac-derived stem cell of claim 7,
2 which on differentiation produces progeny cells including at least two cells selected from
3 the group.

1 9. The isolated nonadherent cardiac-derived stem cell of claim 7,
2 which on proliferation and differentiation produces progeny cell comprising all of the cell
3 types selected from the group.

1 10. The isolated nonadherent cardiac-derived stem cell of claim 7 that
2 is a human stem cell.

1 11. The isolated nonadherent cardiac-derived stem cell of claim 7 that
2 is a mouse stem cell.

1 12. The isolated nonadherent cardiac-derived stem cell of claim 11 that
2 is from a p53-deficient mouse.

1 13. An isolated adherent human cardiac-derived stem cell, which
2 proliferates and differentiates to produce progeny cells comprising a cell type selected
3 from the group consisting of a fibroblast, a smooth muscle cell, skeletal muscle cell, a
4 cardiocyte, a chondrocyte, a keratinocyte and an osteoblast.

1 14. The isolated human adherent cardiac-derived stem cell of claim 13,
2 which proliferates and differentiates to produce progeny cells comprising at least two cell
3 types selected from the group.

1 15. The isolated adherent cardiac-derived stem cell of claim 13, which
2 proliferates and differentiates to produce progeny cells comprising each cell type selected
3 from the group.

1 16. An isolated adherent cardiac-derived stem cell, which proliferates
2 and differentiates to produce progeny cells comprising a cardiocyte and either a
3 chondrocyte or a keratinocyte.

1 17. The isolated adherent cardiac-derived stem cell of claim 16, which
2 proliferates and differentiates to produce progeny cells further comprising one or more

3 cell type selected from the group consisting of a fibroblast, a smooth muscle cell, skeletal
4 muscle cell, and an osteoblast.

1 18. A method of preparing an isolated nonadherent cardiac-derived
2 stem cell, comprising
3 centrifuging a suspension of cells from heart tissue of a subject on a
4 density gradient;
5 isolating a band of cells comprising myocytes;
6 propagating the cells until adherent cardiocytes have died or been
7 discarded leaving suspension cells;
8 culturing the suspension cells until a population of nonadherent cardiac
9 cells is detectable.

1 19. The method of claim 18, further comprising:
2 obtaining heart tissue from a subject;
3 digesting the heart tissue with collagenase to produce the suspension of
4 cells.

1 20. A method of preparing an adherent cardiac-derived stem cell,
2 comprising
3 providing a nonadherent cardiac-derived stem cell of claim 1;
4 propagating the stem cell until adherent progeny cells appears;
5 identifying an adherent cell lacking markers of a cell selected from the
6 group consisting of myoblasts, smooth muscle cells, skeletal muscle cells, cardiocytes,
7 osteoblasts, keratinocytes and chondroblasts, which on proliferation and differentiation
8 produces progeny cells comprising at least one cell type from the group.

1 21. A method of preparing a cardiocyte, comprising
2 providing a nonadherent cardiac-derived stem cell of claim 1;
3 propagating the cell under conditions in which the cell proliferates and
4 differentiates to produce progeny cells comprising adherent cells;
5 identifying an adherent cell with differentiation markers characteristic of a
6 cardiocyte.

1 22. A method of preparing a cardiocyte, comprising
2 providing a nonadherent cardiac-derived stem cell of claim 13 or 16;

- 3 propagating the cell under conditions in which the cell proliferates and
4 differentiates to produce adherent progeny cells;
5 identifying an adherent cell with differentiation markers characteristic of a
6 cardiocyte.

1 23. A method of propagating a nonadherent cardiac-derived stem cell,
2 comprising;
3 culturing the cell in the presence of fibroblast growth factor, wherein the
4 cell propagates.

1 24. An isolated population of cells comprising smooth muscle cells,
2 skeletal muscle cells, cardiocytes, fibroblasts, keratinocytes, osteoblasts and
3 chondrocytes.

1 25. A method of treating a patient suffering from necrotic heart tissue,
2 comprising administering to the patient an effective dose of nonadherent stem cells
3 according to claim 1, whereby the stem cells proliferate and differentiate to produce
4 cardiocytes, which replace the necrotic tissue.

1 26. The method of claim 25, wherein the nonadherent stem cells are
2 administered directly to the heart of the patient.

1 27. The method of claim 25, wherein the nonadherent stem cells are
2 administered intravenously.

1 28. The method of claim 25, further comprising administering FGF to
2 the patient to stimulate proliferation and/or differentiation of the nonadherent cells.

1 29. The method of claim 25, further comprising administering stem
2 cell factor to the patient to stimulate differentiation of the nonadherent cells to
3 cardiocytes.

1 30. The method of claim 18, wherein the patient has a congestive heart
2 defect.

1 31. The method of claim 25, wherein the stem cells are obtained from
2 the blood of the patient, and propagated in vitro before readministering to the patient.

1 32. A method of treating a patient suffering from necrotic heart tissue,
2 comprising administering to the patient an effective dose of adherent stem cells according
3 to claim 13, whereby the stem cells proliferate and differentiate to produce cardiocytes,
4 which replace the necrotic tissue.

1 33. The method of claim 32, wherein the nonadherent stem cells are
2 administered directly to the heart of the patient.

1 34. The method of claim 32, further comprising administering FGF to
2 the patient to stimulate proliferation of the adherent cells.

1 35. The method of claim 32, further comprising administering stem
2 cell factor to the patient to stimulate differentiation of the adherent cells to cardiocytes.

1 36. A method of screening potential agents for activity in promoting
2 proliferation and/or differentiation of cardiac-derived stem cells
3 propagating the nonadherent cardiac-derived stem cell according to claim
4 1 or in claim 6 in the presence of a potential agent,
5 monitoring a change in differentiation state of progeny cells relative to the
6 nonadherent cardiac-derived stem cells.

1 37. The method of claim 36, wherein the change of differentiation state
2 is adhesion of progeny cells.

1 38. The method of claim 36, wherein the monitoring comprising
2 monitoring the appearance of cardiocytes.

1 39. The method of claim 36, wherein the monitoring comprises
2 monitoring the appearance of an adherent cardiac-derived stem cell.

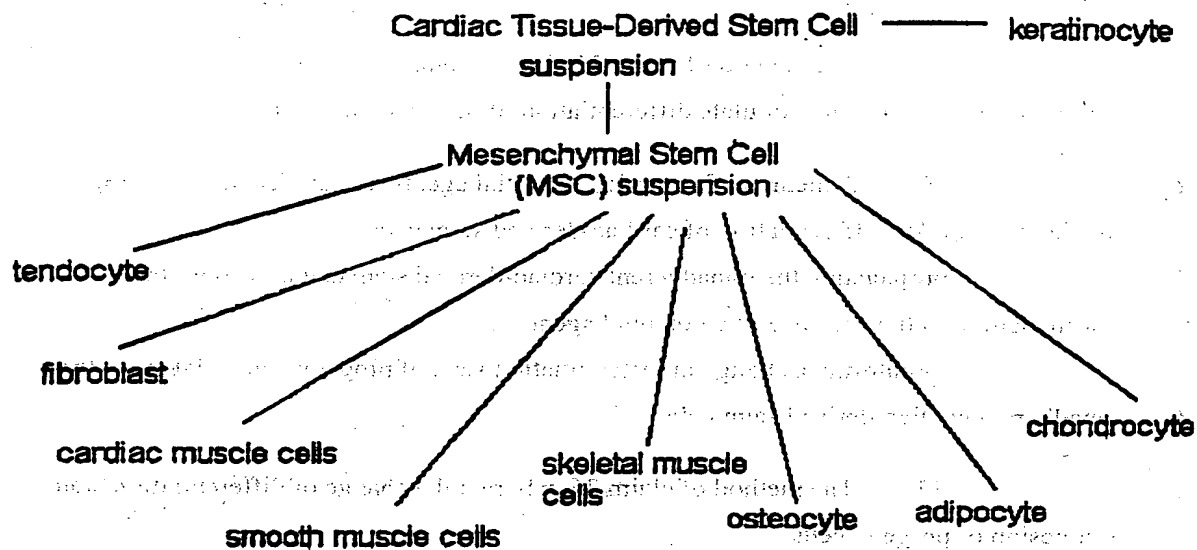
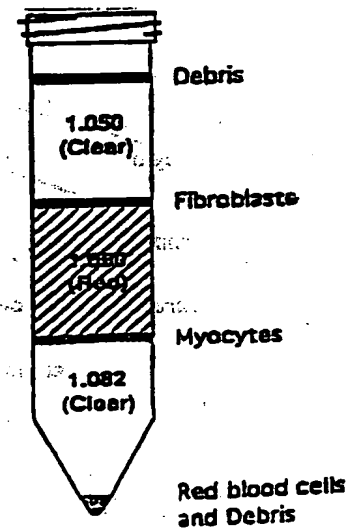
Figure 1

Fig. 2



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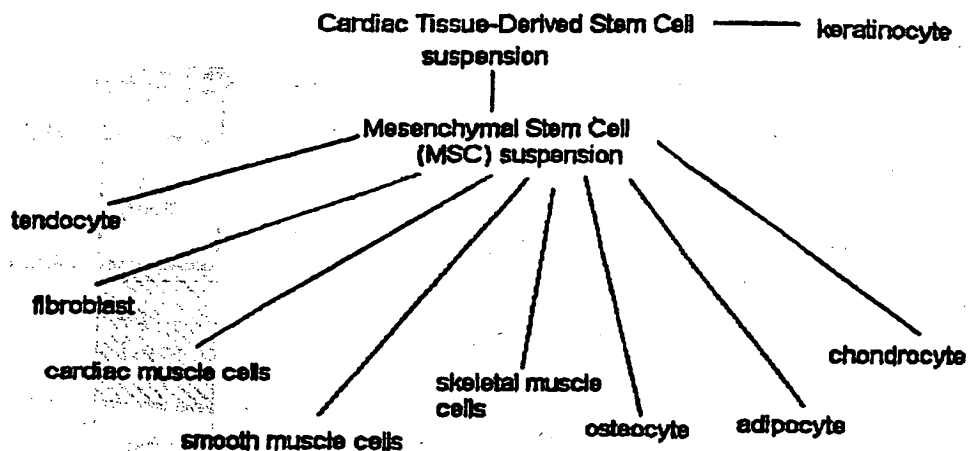
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(54) Title: CARDIAC-DERIVED STEM CELLS



(57) Abstract

The invention provides cardiac-derived pluripotent stem cells, which on proliferation and differentiation can produce a variety of cell types including cardiocytes, fibroblasts, smooth muscle cells, skeletal muscle cells, keratinocytes, osteoblasts and chondrocytes. The cells can be used in methods of treating patients suffering from necrotic heart tissue. The stem cells proliferate and differentiate to produce cardiocytes replacing the necrotic tissue. The cells can also be used to screen compounds for activity in promoting proliferation and/or differentiation of cardiac-derived stem cells.

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06356

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/06 C12N5/08 C12N5/10 A61K35/34 G01N33/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	DE 44 41 327 C (INST PFLANZENGENETIK UND KULTU) 9 November 1995 (1995-11-09) see the whole document, especially column 3 line 25-37	1-11, 13-17, 20-23, 25-30, 32-39
X	LI R K (REPRINT) ET AL: "Natural history of fetal rat cardiomyocytes transplanted into adult rat myocardial scar tissue" CIRCULATION, (4 NOV 1997) VOL. 96, NO. 9, SUPP. 'S', PP. 179-186, XP002118291 the whole document especially p.180 column 1 line 3-10	1-3, 5-11, 13-23, 25-39

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☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 October 1999

Date of mailing of the international search report

03/11/1999

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Stein, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/06356

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DOETSCHMAN T C ET AL: "THE IN VITRO DEVELOPMENT OF BLASTOCYST-DERIVED EMBRYONIC STEM CELL LINES: FORMATION OF VISCERAL YOLK SAC, BLOOD ISLANDS AND MYOCARDIUM" JOURNAL OF EMBRYOLOGY AND EXPERIMENTAL MORPHOLOGY, vol. 87, 1 January 1985 (1985-01-01), pages 27-45, XP000569978 ISSN: 0022-0752 the whole document especially p.33 line 10-p.34 line 3	24
X	SOONPAA MH ET AL: "Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium" SCIENCE, vol. 264, 1 April 1994 (1994-04-01), pages 98-101, XP002118292 see the whole document, especially references 7 and 8 page 101	1-3,5-9, 11, 16-18, 25-35
X	WO 96 38544 A (DIACRIN INC) 5 December 1996 (1996-12-05) page 4, line 20 -page 6, line 19 page 20, line 15 -page 22, line 30	13,32
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A	WO 96 07733 A (MACKAY VIVIAN L ;MOORE EMMA E (US); ZYMOGENETICS INC (US)) 14 March 1996 (1996-03-14) page 4, line 10 -page 5, line 34 page 11, line 18 - line 32 page 15, line 28 -page 16, line 11 page 22, line 33 - line 36 claims 1,2,7,8,16-18	4,11-13, 20-22, 36-39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 99/06356

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 25-35 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
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- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 99/06356

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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